DIABETES THERAPY

The present invention relates to gene therapy and provides stable, transformed mammalian cell lines and vectors useful as vehicles for transferring functional DNA sequences whose protein products are useful in the treatment of *diabetes mellitus*.

The human hormone glucagon is a 29-amino acid hormone produced in pancreatic A-cells. The hormone belongs to a multi-gene family of structurally related peptides that include secretin, gastric inhibitory peptide, vasoactive intestinal peptide and glicentin. These peptides variously regulate carbohydrate metabolism, gastrointestinal mobility and secretory processing. However, the principal recognized actions of pancreatic glucagon are to promote hepatic glycogenolysis and gluconeogenesis, resulting in an elevation of blood sugar levels. In this regard, the actions of glucagon are counter regulatory to those of insulin and may contribute to the hyperglycemia that accompanies *diabetes mellitus* (Lund, P.K., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 79:345-349 (1982)).

When glucagon binds to its receptor on insulin producing cells, cAMP production increases which in turn stimulates insulin expression (Korman, L.Y., *et al.*, *Diabetes*, 34:717-722 (1985)). Moreover, high levels of insulin down-regulate glucagon synthesis by a feedback inhibition mechanism (Ganong, W.F., *Review of Medical Physiology*, Lange Publications, Los Altos, California, p. 273 (1979)). Thus, the expression of glucagon is carefully regulated by insulin, and ultimately by serum glucose levels.

Preproglucagon, the precursor form of glucagon, is encoded by a 360 base pair gene and is processed to form proglucagon (Lund, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 79:345-349 (1982)). Patzelt, *et al.* (*Nature*, 282:260-266 (1979)) demonstrated that proglucagon is further processed into glucagon and a second peptide. Later experiments demonstrated that proglucagon is cleaved carboxyl to Lys-Arg

or Arg-Arg residues (Lund, P.K., *et al.*, Lopez L.C., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 80:5485-5489 (1983), and Bell, G.I., *et al.*, *Nature* 302:716-718 (1983)). Bell, G.I., *et al.*, also discovered that proglucagon contained three discrete and highly homologous peptide regions which were designated glucagon, glucagon-like peptide 1 (GLP-1), and glucagon-like peptide 2 (GLP-2). Lopez, *et al.*, demonstrated that GLP-1 was a 37 amino acid peptide and that GLP-2 was a 34 amino acid peptide. Analogous studies on the structure of rat preproglucagon revealed a similar pattern of proteolytic cleavage at Lys-Arg or Arg-Arg residues, resulting in the formation of glucagon, GLP-1, and GLP-2 (Heinrich, G., *et al.*, *Endocrinol.*, 115:2176-2181 (1984)). Finally, human, rat, bovine, and hamster sequences of GLP-1 have been found to be identical (Ghiglione, M., *et al.*, *Diabetologia*, 27:599-600 (1984)).

The conclusion reached by Lopez, *et al.*, regarding the size of GLP-1 was confirmed by studying the molecular forms of GLP-1 found in the human pancreas (Uttenthal, L.O., *et al. J. Clin. Endocrinol. Metabol.*, 61:472-479 (1985)). Their research showed that GLP-1 and GLP-2 are present in the pancreas as 37 and 34 amino acid peptides respectively.

The similarity between GLP-1 and glucagon suggested to early investigators that GLP-1 might have biological activity. Although some investigators found that GLP-1 could induce rat brain cells to synthesize cAMP (Hoosein, N.M., *et al.*, *Febs Lett.* 178:83-86 (1984)), other investigators failed to identify any physiological role for GLP-1 (Lopez, L.C., *et al. supra*). The failure to identify any physiological role for GLP-1 caused some investigators to question whether GLP-1 was in fact a hormone and whether the relatedness between glucagon and GLP-1 might be artifactual.

It has now been shown that biologically processed forms of GLP-1 have insulinotropic properties and delay gastric emptying. GLP-1(7-34) and GLP-1(7-35) are disclosed in U.S. Patent No: 5,118,666, herein incorporated by

reference. GLP-1(7-37) is disclosed in U.S. Patent No: 5,120,712, herein incorporated by reference.

Variants and analogs of GLP-1 are known in the art. These variants and analogs include, for example, GLP-1(7-36), Gln^{9}-GLP-1(7-37), Thr^{16}-Lys^{18}-GLP-1(7-37), and Lys^{18}-GLP-1(7-37). Derivatives of GLP-1 include, for example, acid addition salts, carboxylate salts, lower alkyl esters, and amides (*see*, e.g., WO91/11457). Generally, the various disclosed forms of GLP-1 are known to stimulate insulin secretion (insulinotropic action) and cAMP formation (*see*, e.g., Mojsov, S., *Int. J. Peptide Protein Research*, 40:333-343 (1992)).

More importantly, numerous investigators have demonstrated a predictable relationship between various *in vitro* laboratory experiments and mammalian, especially human, insulinotropic responses to exogenous administration of GLP-1, GLP-1(7-36) amide, and GLP-1(7-37) acid (*see*, e.g., Nauck, M.A., *et al.*, *Diabetologia*, 36:741-744 (1993); Gutniak, M., *et al.*, *New England J. of Medicine*, 326(20):1316-1322 (1992); Nauck, M.A., *et al.*, *J. Clin. Invest.*, 91:301-307 (1993); and Thorens, B., *et al.*, *Diabetes*, 42:1219-1225 (1993)).

The fundamental defects responsible for causing hyperglycemia in mature onset diabetes include impaired secretion of endogenous insulin and resistance to the effects of insulin by muscle and liver tissue (Galloway, J.S., *Diabetes Care*, 13:1209-1239, (1990)). The latter defect results in excess glucose production in the liver. Thus, whereas a normal individual releases glucose at the rate of approximately 2 mg/kg/minute, a patient with mature onset diabetes releases glucose at a rate exceeding 2.5 mg/kg/minute, resulting in a net excess of at least 70 grams of glucose per 24 hours.

Because there exists exceedingly high correlation between hepatic glucose production, fasting blood glucose levels, and overall metabolic control as indicated by glycohemoglobin measurements (Galloway, J.A., *supra*; and Galloway, J.A., *et al.*, *Clin. Therap.*, 12:460-472 (1990)), it

is readily apparent that control of fasting blood glucose is essential for achieving overall normalization of metabolism sufficient to prevent hyperglycemic complications. Since existing insulin therapies rarely normalize hepatic glucose production without producing significant hyperinsulinemia and hypoglycemia (Galloway, J.A., and Galloway, J.A., *et al.*, *supra*) alternative approaches for diabetic therapy are needed.

Therapy based on administration of longer acting GLP-1 analogs is one such approach. To date however, this approach has failed to deliver long term efficacious doses to individuals due in large part because the serum half-life of GLP-1(7-37) is quite short. Therefore, the quest for alternative approaches continues.

Gene therapy offers a new avenue for treating diseases rooted in hormone deficiencies because it operates as an *in vivo* protein production and delivery system. This is an especially attractive approach since gene therapy also offers the possibility of physiologically regulating the production and secretion of proteins in response to homeostatic mediators within the body.

Gene therapy can be effected in a number of ways. Retroviral-mediated gene transfer was suggested for treating human diseases involving malfunctioning bone marrow. Anderson *et al.*, *Science* 226: 401 (1984). In addition, PCT Publication Number WO93/09222 (May 13, 1993) and U.S. Patent Number 5,399,346 (March 21, 1995) disclose the genetic alteration of primary human cells that are cultured then reintroduced into the body for the treatment of a variety of diseases.

Many heritable diseases such as diabetes result from the absence of a functional gene necessary to provide the animal with an adequate supply of a vital protein. The goal of gene therapy is to deliver a nucleic acid sequence, present on an RNA or DNA vector, which is capable of encoding the desired, therapeutic protein. Although a number of methods exist to deliver the DNA or RNA vector containing the

desired nucleic acid sequence into the target mammalian cells, two procedures termed *ex vivo* and *in vivo* are generally employed.

Ex vivo gene therapy consists of four primary steps: (1) Primary cells (target cells) are removed from the individual in need of therapy; (2) The gene therapy nucleotide sequence is incorporated into the target cell *in vitro*; (3) The transformed cells expressing the incorporated nucleotide sequence encoding the protein of interest are identified, isolated, and expanded; and (4) The transformed cells are reintroduced into the individual.

Ex vivo therapy generally results in the incorporation of the nucleotide sequence encoding the protein of interest into the chromosomal DNA of the target cell. The critical step of *ex vivo* gene therapy is the proper introduction of the nucleotide sequence encoding the desired protein into the target cell. This transfer of DNA can be accomplished by a number of well documented methods such as: calcium phosphate precipitation, electroporation, and adenoviral or retroviral vectors (Current Protocols in Molecular Biology, John Wiley and Sons, 1989; Methods of Cell Biology, 43: 161 - 189, 1994; *Proc. Natl. Acad. Sci.* USA 85: 6460 - 6464, 1985); although, other well known methods are also consistent with this invention.

In vivo gene therapy generally describes the transfer of a desired nucleic acid sequence, located on a transport vector, directly into an individual in need of therapy. This gene therapy approach does not require that the target cells be first removed and manipulated *in vitro*. Once the vector containing the desired nucleotide sequence is introduced into the individual, the vector moves into the nucleus of the target cell and the nucleotide sequence of interest integrates into the chromosomal DNA of the target cell with varying degrees of efficiency. A number of well documented methods exist for introducing nucleic acids into an individual requiring therapy such as direct injection of

DNA and the use of recombinant viral vectors (Gene Therapy, A Handbook for Physicians, Mary Ann Liebert, Inc., 1994).

The present invention provides a method of treating both Type I and Type II diabetics through a different gene therapy approach. A stable mammalian cell line is transformed by a nucleic acid vector such that it secretes a GLP-1(7-37)-based protein, as defined by SEQ ID NO 1, followed by implantation into an individual needing treatment. Once implanted, the GLP-1(7-37)-based protein, in conjunction with high serum glucose levels, causes pancreatic beta cells to produce insulin in non-insulin dependent *diabetes mellitus* (NIDDM) patients and delays gastric emptying in both NIDDM and insulin dependent *diabetes mellitus* IDDM patients.

Accordingly, one embodiment of this invention provides a method of treating Type I or Type II diabetes in a mammal in need thereof comprising implanting a cell line transformed with a vector comprising a promoter driving expression of a DNA sequence encoding a protein of SEQ ID NO 1

His-Xaa^1-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Xaa^2-Gly-Gln-Ala-Ala-Xaa^3-Xaa^4-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-Xaa^5 **(SEQ ID NO 1)**

wherein

Xaa^1 is Ala, Gly, Val, Thr, and Ile;
Xaa^2 is Glu, Gln, Ala, Thr, Ser, and Gly;
Xaa^3 is Lys, and Arg;
Xaa^4 is Glu, Gln, Ala, Thr, Ser, and Gly; and,
Xaa^5 is Gly-OH or is absent;

into said mammal such that it is immunologically isolated from the mammal's immune system and secretes a protein of SEQ ID NO 1 into said patient.

Preferred proteins of SEQ ID NO 1 are those in which Xaa^1 is Ala, Xaa^2 is Glu, Xaa^3 is Lys. Xaa^4 is Glu, and Xaa^5 is Gly-OH. More preferred are those in which Xaa^1 is Ala, Gly, Val, Thr, and Ile; Xaa^2 is Glu, Gln, Ala, Thr, Ser,

and Gly; Xaa^3 is Lys, and Arg; Xaa^4 is Glu, Gln, Ala, Thr, Ser, and Gly; and, Xaa^5 is Gly-OH or is absent. Another preferred group are those in which Xaa^1 is Val and Xaa^3 is Lys. A further preferred group of proteins are those wherein Xaa^1 is Ala or Val, Xaa^2 Glu, Xaa^3 is Lys or Arg, Xaa^4 is Glu, and Xaa^5 Gly-OH or is absent. Yet another preferred group are those in which Xaa^1 is Ala, Xaa^2 Glu, Xaa^3 is Lys, Xaa^4 is Glu, and Xaa^5 Gly-OH. Still another preferred group is when Xaa^1 is Val, Xaa^2 Glu, Xaa^3 is Lys, Xaa^4 is Glu, and Xaa^5 Gly-OH.

Nucleotide sequences encoding any one of the polypeptides of SEQ ID NO 1 may be prepared by a variety of means readily apparent to those skilled in the art. Wholly synthetic nucleotide sequences or semi-synthetic sequences derived in part from a natural GLP-1 gene may be used. Owing to the natural degeneracy of the genetic code, the skilled artisan will recognize that a sizable yet definite number of nucleotide sequences may be constructed which encode the proteins of SEQ ID NO 1. A synthetic DNA sequence encoding a GLP-1-based protein of SEQ ID NO 1 may be prepared by techniques well known in the art in substantial accordance with the teachings of Brown, et al. (1979) *Methods in Enzymology*, Academic Press, N.Y., Vol. 68, pgs. 109-151. The DNA sequence may be generated using conventional DNA synthesizing apparatus such as an Applied Biosystems Model 380A or 380B DNA synthesizer (commercially available from Applied Biosystems, Foster City, California). Commercial services are also available for the construction of such nucleotide sequences based on the amino acid sequence.

In one preferred embodiment of the invention as exemplified herein, the coding sequence for a protein of SEQ ID NO 1 is the following:

5' - CAT GCT GAA GGG ACC TTT ACC AGT GAT GTA AGT TCT TAT TTG GAA GGC CAA GCT GCC AAG GAA TTC ATT GCT TGG CTG GTG AAA GGC CGA GGA - 3' **(SEQ ID NO 2).**

This sequence encodes the following protein of SEQ ID NO 1:

H_2N- His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val Lys Gly Arg Gly -OH **(SEQ ID NO 3).**

In another preferred embodiment of the invention as exemplified herein, the coding sequence for a protein of SEQ ID NO 1 is the following:

5' - CAT GTT GAA GGG ACC TTT ACC AGT GAT GTA AGT TCT TAT TTG GAA GGC CAA GCT GCC AAG GAA TTC ATT GCT TGG CTG GTG AAA GGC CGA GGA - 3' **(SEQ ID NO 4).**

This sequence encodes the following protein of SEQ ID NO 1:

H_2N- His Val Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val Lys Gly Arg Gly -OH **(SEQ ID NO 5).**

Coding regions for SEQ ID NO 1 may be fused to a leader sequence which signals the cell to secrete a precursor peptide of SEQ ID NO 1 that is subsequently processed by the cell during the secretion process to a protein of SEQ ID NO 1. Many such leader sequences are know in the art. One well known and preferred leader sequence is the hybrid tissue plasminogen activator/protein C prepropeptide described in Berg *et al. Biochem. Biophys. Res. Commun.* 179: 1289-1296 (1991). Typically nucleotide sequences encoding precursor peptides of SEQ ID NO 1 are flanked by linker DNA to facilitate enzymatic ligation into expression vectors as is later exemplified herein.

Once a suitable coding sequence of SEQ ID NO 1 is constructed and optionally fused and flanked by an appropriate leader sequence and linker DNA, the construct is ligated into an expression vector which is then introduced into an appropriate cell line. Construction of suitable vectors containing the desired coding and control sequences may be constructed by standard ligation techniques. Isolated

plasmids or nucleotide fragments are cleaved, tailored, and religated in the manner necessary to achieve the desired plasmids.

To effect the expression of a polypeptides of SEQ ID NO 1, one ligates a nucleotide sequence encoding the polypeptide into an appropriate recombinant nucleotide expression vector through the use of appropriate enzymes. The nucleotide sequence encoding a polypeptide of SEQ ID NO 1 is designed to possess restriction endonuclease cleavage sites at either end of the DNA to facilitate isolation from and integration into these amplification and expression plasmids. The coding sequence may be readily modified by the use of synthetic linkers to facilitate the incorporation of the coding sequence into the desired cloning vectors by techniques well known in the art. The particular endonucleases employed will be dictated by the restriction endonuclease cleavage pattern of the parent expression vector to be employed. The choice of restriction sites are chosen so as to properly orient the coding sequence such that it is properly associated with the promoter and ribosome binding site of the expression vector, both of which are functional in the host cell in which a compound of SEQ ID NO 1 is to be expressed.

In addition to the desired nuceotide sequence which will code for the therapeutic protein, the expression vector may contain several other functional elements. One such element is the promoter and upstream regulatory sequences which control the level of expression of the protein of interest. Some expression vectors contain promoters and regulatory sequences which normally regulate transcription of cellular genes. One such promoter is the mouse metallothionein-I promoter which has been shown to function both *in-vitro* and *in-vivo* (Palmiter et al., *Nature* **300**: 611 - 615, 1982). In addition, promoters and regulatory sequences from viruses are frequently used in expression vectors (Dijkema et al., *EMBO J.* 4, 471, 1985; Gorman et al., *Proc. Natl. Acad. Sci.* 79: 6777, 1982; Boshart, et al. *Cell* 41:

521, 1985). Although, Verma *et al.* have shown that the retrovirus promoter and enhancer sequences do not function for long periods of time *in-vivo*. In addition, the expression vector also carries an origin of replication as well as marker sequences which are capable of providing phenotypic selection in transformed cells.

Because the proteins useful in the present invention do not require post-translational processing mechanisms other than enzymatic removal of the propeptide leader sequence, many stable human cell lines are consistent with the practice of the invention. One preferred cell line is the human embryonal kidney cell line 293, available from the permanent collection of the American Type Culture Collection.

A number of well known methods exist for introducing the genetic material into target cells such as chemical (calcium phosphate precipitation), physical (electorporation and microinjection), and viral methods (adenovirus, retrovirus, and adeno-associated virus) (Methods for Gene Transfer, Gene Therapy, Mary Ann Liebert, Inc., 1994). All such methods are consistent with the practice of the present invention. The techniques of transforming mammalian cells with the aforementioned vector types are well known in the art and may be found in such general references as Maniatis, et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York or Current Protocols in Molecular Biology, Vol. 1, (1988), Wiley Interscience, and supplements.

Stable transformed cell lines that express proteins of SEQ ID NO 1 must then be implanted into the individual in need of such treatment. Because such transformed cell lines generally will be histologically incompatible with the individuals receiving them, the cells must to be protected from the recipient's immune system. Once way of protecting the implanted cells is by masking them with $F(ab')_2$ fragments specific for HLA class I antigens. Immunological masking

methods are well known in the art. For example, see Faust *et al. Science* 252:1700-1702 (1991). Other means for protecting the implanted cells from the recipient's immune system are consistent with this invention. Such methods include but are not limited to encapsulation in semi-permeable membranes (Lanza et al. *Diabetes* 41: 1503 - 1510) and through the use of immunosuppressants (Rynasiewicz et al., *Diabetes* 31: 92 - 108, 1982).

By way of illustration, the following examples are provided to help describe how to make and practice the various embodiments of the invention. These examples are in no way meant to limit the scope of the invention.

Example 1

Construction of Intermediate

Plasmid pLP53-tLB+GLP-1

A. Preparation of BglII-Mung Bean-AvrII Digested pLP53-tLB

The plasmid pLP53-tLB was isolated from *E. coli* K12 AG1 (on deposit under terms of the Budapest Treaty and made part of the permanent stock culture collection of the Northern Regional Research Laboratories (NRRL), agricultural Research Service, U.S. Dept. of Agriculture, Peoria, IL 61604 under accession number NRRL B-18714) using the Plasmid Purification Midi Kit. (Qiagen, Inc., 9600 DeSoto Avenue, Chatsworth, CA 91311).

Sixty µl (approximately 20 µg) of pLP53-tLB DNA was digested with 2 µl (20 units) of BglII in a 70 µl reaction volume containing 50mM Tris-HCl (pH 8.0), 10mM $MgCl_2$, and 100mM NaCl. The sample was incubated at 37°C for one hour. 17.5 µl of 5x stop mix (25% glycerol, 2% SDS, 0.05% bromophenol blue, 0.05% xylene cyanol in water) was added and then the reaction was heated at 70°C for 15-20 minutes to inactivate the restriction enzyme. The mixture was spin dialyzed using G-50 Sephadex Quick Spin columns (Boehringer Mannheim Corporation, P.O. Box 50414, 9115 Hague Road,

Indianapolis, IN 46250-0414) to remove the reaction components.

The 5' protruding ends created by cleavage with BglII were removed using Mung Bean Nuclease. The BglII digested pLP53-tLB DNA was incubated with 0.3 µl (approximately 3.3 units) of Mung Bean Nuclease in a 100 µl reaction volume containing 10mM Tris-HCl (pH 7.9 at 25°C), 10mM $MgCl_2$, 50mM NaCl, 1mM DTT, and 1mM $ZnSO_4$. The reaction was allowed to proceed for 30 minutes at 30°C. One µl of 1% SDS was added to inactivate the nuclease. Due to an incomplete BglII digest, the digested and undigested DNA was separated by gel electrophoresis. Ten µl of gel loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in water) was added to the reaction. The reaction was loaded into the preparative well of a 1.5% NuSieve GTG agarose (FMC Bioproducts, 191 Thomaston Street, Rockland, ME 04841)/TAE buffer gel and then electrophoresed. The gel was stained with ethidium bromide and the DNA was visualized by ultraviolet light. The digested DNA band was excised with a scalpel and placed into two micro-tubes. The DNA was purified from the low melting point agarose using the Wizard PCR Preps DNA Purification System (Promega, 2800 Woods Hollow Road, Madison, WI 53711-5399).

One hundred µl of BglII-Mung Bean digested pLP53-tLB DNA was further digested with 4 µl (approximately 16 units) of AvrII in a reaction volume of 120 µl containing 10mM Tris-HCl (pH 7.9 at 25°C), 10mM $MgCl_2$, 50mM NaCl, 1mM DTT. The sample was incubated at 37°C for 30 minutes. To prevent recircularization, the BglII-Mung Bean-AvrII digested pLP53-tLB DNA was dephosphorylated (removal of 5' phosphate groups) by the addition of 2 µl (2 units) of calf intestinal alkaline phosphatase to the reaction. The sample was incubated at 37°C for an additional 30 minutes. Twenty-four µl of 5x stop mix was added. The sample was heated at 70°C for 15-20 minutes to inactivate the enzymes and then spin dialyzed using G-50 Sephadex Quick Spin columns (Boehringer Mannheim Corporation, P.O. Box 50414, 9115 Hague Road,

Indianapolis, IN 46250-0414) in order to remove the AvrII produced small DNA fragments. The DNA was precipitated by addition of 0.1 volume of 3M sodium acetate (pH 5.2) and 2.5 volumes of absolute ethanol. This mixture was mixed thoroughly and then chilled to -20°C. The precipitate was collected by centrifugation for 30 minutes. The supernatant was discarded and the pellet was washed with 700 µl of cold 70% ethanol. The sample was centrifuged for 15 minutes. The supernatant was discarded, the DNA pellet was dried and then resuspended in 25 µl of water.

B. Preparation of GLP-1 Linker

The following single stranded DNA segments were conventionally synthesized by methods well known in the art on an automated DNA synthesizer (model 394 Applied Biosystems 850 Lincoln Center Drive, Foster City, CA 94404-1128) using ß-cyanoethyl phosphoramidite chemistry.

GLP-1.1

5' - GACATGCTGA AGGGACCTTT ACCAGTGATG TAAGTTCTTA TTTGGAAGGC CAAGCTGCCA AGGAATTCAT TGCTTGGCTG GTGAAAGGCC GAGGATAGGG ATCCC - 3' **(SEQ ID NO 6)**

GLP-1.2

5' - CTAGGGGATC CCTATCCTCG GCCTTTCACC AGCCAAGCAA TGAATTCCTT GGCAGCTTGG CCTTCCAAAT AAGAACTTAC ATCACTGGTA AAGGTCCCTT CAGCATGTC - 3' **(SEQ ID NO 7)**

GLP-1.1 and GLP-1.2 are complementary DNA molecules. The synthetic DNA molecules were dissolved in water and stored at less than 0°C.

To anneal the DNA strands, approximately 92.7 pmoles each of GLP-1.1 and GLP-1.2 were mixed in 50mM Tris-HCl (pH 7.4) and 10mM $MgCl_2$ in a total volume of 80 µl and boiled for 5 minutes. The mixture was slowly brought to room temperature and then transferred to 4°C overnight. This process allowed the two complementary strands to anneal and

form the double stranded DNA linker known as GLP-1. The linker was stored at -20°C. In order to be able to ligate into the dephosphorylated BglII-Mung Bean-AvrII digested pLP53-tLB DNA segment, the GLP-1 linker must have 5' phosphate groups. The phosphate groups were added by the use of the enzyme T4 polynucleotide kinase. The kinase reaction contained 80 µl of the GLP-1 linker, 0.33µM ATP, 70mM Tris-HCl (pH 7.6), 10mM $MgCl_2$, 100mM KCl, 1mM ß-mercaptoethanol and 37.2 µl (372 units) of T4 polynucleotide kinase. The reaction was incubated at 37°C for 30 minutes. Sixteen µl of 500mM EDTA was added to stop the reaction. The reaction was extracted once with a mixture of phenol:chloroform:isoamyl alcohol (25:24:1) followed by an extraction with chloroform:isoamyl alcohol (24:1). One hundred µl of the aqueous layer was spin dialyzed using G-50 Sephadex Quick Spin columns (Boehringer Mannheim) in order to remove the reaction components. The DNA was precipitated by addition of 0.1 volume of 3M sodium acetate (pH 5.2), 0.1 volume of 100mM $MgCl_2$ and 2.5 volumes of absolute ethanol. This mixture was mixed thoroughly and then chilled at -20°C. The precipitate was collected by centrifugation for 30 minutes. The supernatant was discarded and the pellet was washed with 700 µl of cold 70% ethanol. The sample was centrifuged for 15 minutes. The supernatant was discarded, the DNA pellet was dried and then resuspended in 10 µl of water.

C. Final Construction of pLP53-tLB+GLP-1

The DNA prepared in Example 1A was ligated with linker GLP-1. Two µl of DNA prepared in Example 1A and 4 µl of GLP-1 linker were ligated in a reaction that contained 2 µl (2 units) of T4 DNA ligase, 50mM Tris-HCl (pH 7.6), 10mM $MgCl_2$, 1mM ATP, 1mM DTT, and 50% (w/v) polyethylene glycol-8000 in a total volume of 10 µl. The mixture was incubated at 16°C for 16 hours. The ligation was used to transform *E. coli* K12 INVαF' cells as generally described below.

D. Transformation Procedure

Frozen competent *E. coli* K12 INVαF' cells were obtained from Invitrogen (3985 B Sorrento Valley Boulevard, San Diego, CA 92121). Two µl of 0.5M ß-mercaptoethanol were added to 50 µl of thawed competent cells. About 1-2 µl of the ligation reaction was mixed with the cells. The cell-DNA mixture was incubated on ice for 30 minutes, heat-shocked at 42°C for exactly 30 seconds and then chilled on ice for 2 minutes. The cell-DNA mixture was diluted into 450µl of SOC media (2% tryptone, 0.05% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose in distilled water) and incubated at 37°C for one hour in a rotary shaker set at about 225 rpm. Aliquots of up to 200 µl were plated on TY-agar plates (1% tryptone, 0.5% yeast extract, 1% NaCl, and 1.5% agar, pH 7.4) containing 100µg/ml ampicillin and then incubated at 37°C until colonies appear.

E. DNA Isolation

Following transformation, ampicillin resistant cells were picked and inoculated into 3 ml of TY broth (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.4) containing 100µg/ml ampicillin. These cultures were grown for about 16 hours at 37°C with aeration. Plasmid DNA was isolated from cultures using Wizard Minipreps obtained from Promega (2800 Woods Hollow Road, Madison, WI 53711-5399). Recombinant plasmids were identified by digestion with restriction endonucleases followed by gel electrophoresis analysis.

To obtain larger amounts of pLP53-tLB+GLP-1 plasmid DNA, large scale isolation was performed using the Plasmid Purification Midi Kit (Qiagen, Inc.).

Example 2

Construction of pGT-h+tLB+GLP-1

A. Preparation of BclI Digested pGT-h

The plasmid pGT-h was isolated from *E.coli* K12 GM48 (on deposit under terms of the Budepest Treaty and made part

of the permanent stock culture collection of the NRRL under accession number B-18592 using the Plasmid Purification Midi Kit (Qiagen, Inc.).

Ten µg (37.5 µl) of pGT-h DNA was digested to completion with 2 µl (20 units) of BclI in a 45 µl reaction volume containing 50mM Tris-HCl (pH 8.0), 10mM $MgCl_2$, 50mM NaCl. The sample was incubated at 50°C for 1 hour. Eleven µl of 5x stop mix was added to the reaction mixture. The mixture was heated at 70°C for 15-20 minutes to inactivate the restriction enzyme and then spin dialyzed using G-50 Sephadex Quick Spin columns (Boehringer Mannheim) in order to remove the reaction components. The DNA was precipitated by adding 0.1 volume of 3M sodium acetate (pH 5.2) and 2.5 volumes of absolute ethanol. This mixture was mixed thoroughly and then chilled at -20°C. The precipitate was collected by centrifugation for 30 minutes. The supernatant was discarded and the pellet was washed with 700 µl of cold 70% ethanol. The sample was centrifuged for 15 minutes. The supernatant was discarded, the DNA pellet was dried and then resuspended in 20 µl of water.

Calf intestinal alkaline phosphatase was used to remove the 5' phosphate groups from the DNA segment in order to prevent recircularization of the pGT-h. Ten µl of the BclI digested pGT-h DNA was treated with 1 µl (1 unit) of calf intestinal alkaline phosphatase in a 15 µl reaction containing 50mM Tris-HCl (pH 8.5 at 20°C) and 0.1mM EDTA. The reaction was allowed to proceed for 45 minutes at 37°C. The phosphatase was inactivated by the addition of 1µl of 500mM EDTA and then heating at 65°C for 10 minutes. The reaction volume was increased to 100µl with water and then extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) followed by extraction with chloroform: isoamyl alcohol (24:1). The DNA was recovered from the aqueous layer by the addition of 0.1 volumes of 3M sodium acetate (pH 5.2) and 2.5 volumes of absolute ethanol. The mixture was mixed thoroughly and then chilled at -20°C. The precipitate was collected by centrifugation for 30 minutes. The supernatant

was discarded and the pellet was washed with 700 µl of cold 70% ethanol. The sample was centrifuged for 15 minutes. The supernatant was discarded, the DNA pellet was dried and then resuspended in 25 µl of water.

B. Preparation of pLP53-tLB+GLP-1 BamHI Fragment

Thirty-five µl (10.6 µg) of pLP53-tLB+GLP-1 DNA, prepared in Example 1, was digested with 0.5 µl (25 units) of BamHI in a 40 µl reaction volume containing 50mM Tris-HCl (pH 8.0), 10mM $MgCl_2$, and 100mM NaCl. The reaction was allowed to proceed at 37°C for one hour. Five µl of gel loading dye was added to the reaction. The reaction was loaded into the preparative well of a 4% NuSieve GTG agarose/TAE buffer gel. The DNA was electrophoresed for about one hour at 70 constant volts. The gel was stained with ethidium bromide and then the DNA was visualized by ultraviolet light. The desired 213 base pair DNA band was excised using a scalpel. The DNA was purified from the low melting point agarose using Wizard PCR preps (Promega).

C. Final Construction of pGT-h+tLB+GLP-1

The DNA prepared in Example 2A was ligated with DNA prepared in Example 2B. One µl of DNA from Example 2A and 10.5 µl of DNA from Example 2B were ligated in a reaction that contained 2 µl (2 units) of T4 DNA ligase, 50mM Tris-HCl (pH 7.6), 10mM $MgCl_2$, 1mM ATP, 1mM DTT, and 50% (w/v) polyethylene glycol-8000 in a total volume of 20 µl. The mixture was incubated at 16°C for 16 hours. The ligation reaction was used to transform *E. coli* K12 INVαF' as described in Example 1D. Plasmid DNA was isolated from ampicillin resistant cultures as described in Example 1E.

To obtain larger amounts of pGT-h+tLB+GLP-1 plasmid DNA for the purpose of transfection of mammalian cells, large scale isolation was performed using the alkaline lysis method.

Example 3
Construction of pGT-h+tLB+Val8GLP-1

The plasmid pGT-h+tLB+Val8GLP-1 was constructed substantially in accordance with Examples 1 and 2. To accommodate the change in the amino acid sequence (Ala to Val at Xaa^1 in SEQ ID NO 1) the following coding sequences were substituted for those described in Example 1B:

Val8GLP-1.1

5' - GACATGTTGA AGGGACCTTT ACCAGTGATG TAAGTTCTTA TTTGGAAGGC CAAGCTGCCA AGGAATTCAT TGCTTGGCTG GTGAAAGGCC GAGGATAGGG ATCCC - 3' **(SEQ ID NO 8)**

Val8GLP-1.2

5' - CTAGGGGATC CCTATCCTCG GCCTTTCACC AGCCAAGCAA TGAATTCCTT GGCAGCTTGG CCTTCCAAAT AAGAACTTAC ATCACTGGTA AAGGTCCCTT CAACATGTC - 3' **(SEQ ID NO 9)**

Example 4
Construction of Intermediate Plasmid pM100-neo

A. Preparation of EcoRI Digested pM100

The plasmid pM100 (pOK12) was isolated from *E. coli* K12 RR1ΔM15 using Magic Minipreps (Promega). See Vieira and Messing, *Gene* 100:189-94, 1991). Forty µl of pM100 DNA was digested to completion with 3 µl of EcoRI in a reaction volume of 50 µl containing 50mM Tris-HCl (pH 8.0), 10mM $MgCl_2$, and 100mM NaCl. The sample was incubated at 37°C for 2 hours. The digested DNA was precipitated with ethanol. The final DNA pellet was resuspended in 30 µl of water.

B. Preparation of pBK-neo EcoRI Fragment

The plasmid pBK-neo 1 (described in U S Patent No: 5,550,036, herein incorporated by reference, and available from the American Type Culture Collection under terms of the

Budapest Treaty via Accession No. Atcc 37224) was isolated from *E.coli* K12 HB-1 using the large scale alkaline lysis method. Three µl (26.3 µg) of pBK-neo DNA was digested to completion with 7 µl (15 units) of EcoRI in a reaction volume of 200 µl containing 50mM Tris-HCl (pH 8.0), 10mM $MgCl_2$, and 100mM NaCl. The sample was incubated at 37°C. Fifty µl of the reaction was loaded into the preparative well of an agarose/TAE buffer gel and then electrophoresed. The desired 4.2 Kb DNA band was isolated from the agarose by electrophoresing onto DEAE-cellulose membrane. Following elution from the DEAE-cellulose membrane, the DNA was precipitated with ethanol. The final DNA pellet was resuspended in 30 µl of water.

C. Final Construction of pM100-neo

The DNA prepared in Example 4A was ligated with DNA prepared in Example 4B. Two µl of DNA from Example 4A and 2 µl of DNA from Example 4B were ligated in a reaction that contained 1 µl (1 unit) of T4 DNA ligase, 50mM Tris-HCl (pH 7.6), 10mM $MgCl_2$, 1mM ATP, 1mM DTT, and 50% (w/v) polyethylene glycol-8000 in a total volume of 16 µl. The mixture was incubated at 16°C for 16 hours. Ten µl of the ligation reaction was used to transform *E. coli* K12 RR1ΔM15 as described in Example 5D. Aliquots of up to 200 µl were plated on TY-agar plates (1% tryptone, 0.5% yeast extract, 1% NaCl, and 1.5% agar, pH 7.4) containing kanamycin and then incubated at 37°C until colonies appear.

D. DNA Isolation

Following transformation, kanamycin resistant cells were picked and inoculated into 5 ml of TY broth (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.4) containing kanamycin. These cultures were grown for about 8 hours at 37°C with aeration. Plasmid DNA was isolated from cultures using Magic Minipreps obtained from (Promega). Recombinant plasmids were identified by digestion with restriction endonucleases followed by gel electrophoresis analysis.

To obtain larger amounts of pM100-neo plasmid DNA, large scale isolation was performed using the Plasmid Purification Kit (Qiagen, Inc.).

Example 5

Construction of pMT-h+tLB+Val8GLP-1

A. Preparation of Bst1107I-BclI Digested pGT-h

The plasmid pGT-h was isolated as described in substantial accordance with Example 2A. Three µg (4.6 µl) of pGT-h DNA was digested to completion with 1 µl (8 units) of Bst1107I in a 10 µl reaction volume containing 10mM Tris-HCl, 10mM $MgCl_2$, 100mM KCl (pH 8.5 at 37°C). The sample was incubated at 37°C for 1 hour. The Bst1107I digested pGT-h DNA was purified from the reaction components using the Wizard DNA Clean-Up System (Promega). The Bst1107I digested pGT-h DNA was concentrated in a 6.7 µl volume using a microcon 50 (Amicon, Inc. 72 Cherry Hill Drive, Beverly, MA 01915).

The 6.7 µl of Bst1107I digested pGT-h DNA was further digested with 1 µl (10 units) of BclI in a reaction volume of 10 µl containing 50mM Tris-HCl (pH 8.0), 10mM $MgCl_2$, 50mM NaCl. The sample was incubated at 50°C for 1 hour. To prevent any possible recircularization, the Bst1107I-BclI digested pGT-h was dephosphorylated (removal of 5' phosphate groups) by the addition of 1 µl (1 unit) of calf intestinal phosphatase to the sample. The sample was incubated at 37°C for 30 minutes. Gel loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in water) was added to the reaction. The reaction was loaded into the preparative well of a 1% SeaPlaque GTG agarose/TAE buffer gel and then electrophoresed. The gel was stained with ethidium bromide and then the DNA was visualized by ultraviolet light. The desired 6.2 Kb DNA band was excised with a scalpel and placed into a micro-tube. The DNA was purified from the low melting point agarose using the Wizard PCR Preps DNA Purification System (Promega).

B. Preparation of pM100-neo EcoRV-BglII Fragment

Five µg (3.8 µl) of pM100-neo DNA constructed in Example 4 was digested to completion with 1 µl (10 units) of EcoRV in a 10 µl reaction volume containing 50mM Tris-HCl (pH 8.0), 10mM $MgCl_2$, 50mM NaCl. The reaction was incubated at 37°C for 1 hour. The EcoRV digested pM100-neo was further digested with 1 µl (10units) of BglII in a 20 µl reaction volume containing 50mM Tris-HCl (pH 8.0), 10mM $MgCl_2$, 50mM NaCl. The sample was incubated at 37°C for 1 hour. Gel loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in water) was added to the reaction. The reaction was loaded into the preparative well of a 1% SeaPlaque GTG agarose/TAE buffer gel and then electrophoresed. The gel was stained with ethidium bromide and then the DNA was visualized by ultraviolet light. The desired 1.8 Kb DNA band was excised with a scalpel and placed into a micro-tube. The DNA was purified from the low melting point agarose using the Wizard PCR Preps DNA Purification System (Promega).

C. Final Construction of pMT-h+tLB+Val8GLP-1

The DNA prepared in Example 5A was ligated with the DNA prepared in Example 5B and the DNA prepared in Example 3 (pLP53-tLB+Val8GLP-1 BamHI fragment). Four µl of DNA from Example 5A, 3 µl of DNA from Example 5B and 12 µl of DNA prepared in Example 3 (pLP53-tLB+Val8GLP-1 BamHI fragment) were ligated in a reaction that contained 1 µl (1 unit) of T4 DNA ligase, 50mM Tris-HCl (pH 7.6), 10mM $MgCl_2$, 1mM ATP, 1mM DTT, and 50% (w/v) polyethylene glycol-8000 in a total volume of 25 µl. The mixture was incubated at 16°C for 16 hours.

Frozen competent *E.coli* K12 DH5α cells were transformed using about 3-4 µl of the ligation reaction in substantial accordance with Example 1D, and the plasmid DNA was isolated in substantial accordance with Example 1E.

Example 6

Construction of Stable Cell lines

Plasmid DNA, either pGT-h+tLB+GLP-1, pGT-h+tLB+val8GLP-1, or pMT-h+tLB+val8GLP-1 was transfected into human embryonic kidney 293 cells using the stable $CaPO_4$ method contained in the Mammalian Transfection Kit available from Stratagene (11011 North Torrey Pines Road, LaJolla, CA 92037). Selection was achieved by the addition of 300µg/ml of hygromycin B (Eli Lilly and Company Indianapolis, IN 46285) to the culture medium. Monoclonal cell lines were expanded and screened for the ability to secrete the corresponding protein of SEQ ID NO 1 into the culture medium. The presence of biologically active GLP-1(7-37)-based protein in the culture medium was determined by measuring the amount of luciferase enzyme present in a biological system that expressed luciferase enzyme following stimulation with GLP-1.

Example 7

Implantation

The transformed 293 cell were cultured then surgically transplanted under the kidney capsule of 8 week old Zucker Diabetic Fatty (ZDF/Gmi™-fa/fa) male rats. Under isofurane anesthesia, a dorsal incision was made just posterior to the diaphram, and using a rib spreader, the kidney was exposed. Approximately 20 million transformed 293 cells, in 200 µl of Hank's buffer, were injected just under the kidney capsule using a 23 gauge blunt needle. The incision was sutured and protected from chewing with wound clips.